BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

THIS PAGE BLANK (USPTO)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/00, C07H 15/12, 17/00
A61K 31/70

A1

(11) International Publication Number: WO 93/21309

(43) International Publication Date: 28 October 1993 (28.10.93)

(21) International Application Number: PCT/US93/02913

(22) International Filing Date: 29 March 1993 (29.03.93)

(30) Priority data:
07/866,677 10 April 1992 (10.04.92) US

(71)(72) Applicants and Inventors: LEE, Nancy, M. [US/US]; 1830 Funston Avenue, San Francisco, CA 94116 (US). LOH, Horace, H. [US/US]; 54 Mendosa, San Francisco, CA 94116 (US). LIPPMAN, David [US/US]; 1739 West Sonoma Avenue, Stockton, CA 95204 (US).

(74) Agents: SIEBERT, J., Suzanne et al.; Majestic, Parsons, Siebert & Hsue, Four Embarcadero Center, Suite 1450, San Francisco, CA 94111-4121 (US).

(81) Designated States: AU, CA, JP, NO, RU, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
With amended claims.

(54) Title: OLIGONUCLEOTIDE SEQUENCES AND TRANSGENIC ANIMALS TRANSFECTED THEREWITH HAVING REDUCED SENSITIVITY TO NARCOTIC ANALGESICS

(57) Abstract

Clones (SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3) have been isolated and sequenced. An antisense construct has been made from part of SEQ ID NO:1 that blocks opioid binding in cells and has been used to produce transgenic animals having a reduced sensitivity to narcotic analgesics. The oligonucleotide sequence introduced into the animals at an embryonic stage was the first about 500 base pairs, but was reversed in direction. Oligonucleotide constructs of the invention are also useful as probes or as therapeutic and diagnostic agents.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**MR**	Mauritania
AU	Australia	**GA**	Gabon	**MW**	Malawi
BB	Barbados	**GB**	United Kingdom	**NL**	Netherlands
BE	Belgium	**GN**	Guinea	**NO**	Norway
BF	Burkina Faso	**GR**	Greece	**NZ**	New Zealand
BG	Bulgaria	**HU**	Hungary	**PL**	Poland
BJ	Benin	**IE**	Ireland	**PT**	Portugal
BR	Brazil	**IT**	Italy	**RO**	Romania
CA	Canada	**JP**	Japan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SK**	Slovak Republic
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SN**	Senegal
CM	Cameroon	**LI**	Liechtenstein	**SU**	Soviet Union
CS	Czechoslovakia	**LK**	Sri Lanka	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**MC**	Monaco	**UA**	Ukraine
DK	Denmark	**MG**	Madagascar	**US**	United States of America
ES	Spain	**ML**	Mali	**VN**	Viet Nam
FI	Finland	**MN**	Mongolia		

OLIGONUCLEOTIDE SEQUENCES AND TRANSGENIC ANIMALS TRANSFECTED THEREWITH HAVING REDUCED SENSITIVITY TO NARCOTIC ANALGESICS

Field of the Invention

This invention generally relates to opioid receptors, and more particularly to oligonucleotide constructs that can be used to produce a transgenic non-human animal, such as rodents, with reduced sensitivity to narcotic analgesics, used as probes or used in therapeutic treatment and diagnoses of narcotic addicts.

This invention was made with government support under Grant Nos. DA00564, DA02643, DA01583, awarded by the National Institutes of Health. The government has certain rights in this invention.

Background of the Invention

A μ-opioid-specific receptor protein was purified to apparent homogeneity with a molecular weight of 58,000 daltons in 1986. Cho et al., *Proc. Natl. Acad. Sci. USA*, *83* (1986), pp. 4138-4142. However, this protein from rat brain exhibited high binding activity only when it was reconstituted with certain lipids. The role of the lipids is presumably to stabilize a particular conformation of the protein. Hasegawa et al., *J. Neurochem.*, *49*:4 (1987), pp. 1007-1012. Polyclonal antibodies were reported as having been raised against the purified opioid receptor from bovine brain. Roy et al., *Biochem. & Biophy. Res. Comm.*, *150*:1,

(1988), pp. 237-244. Monoclonal antibodies were raised against the purified opioid receptor protein, and data suggested that the protein may have significant affinity for μ and δ, and to a certain extent, against κ ligands. Roy et al., *Biochem. & Biophy. Res. Comm.*, *154*:2 (1988), pp. 688-693; Roy et al., in *Biochemical Pharmacology -- A Tribute to B.B. Brodie*, ed. Costa, E., New York: Raven Press, Ltd. (1989), pp. 177-188.

In 1989, the purified opioid-binding protein from bovine brain was characterized by cDNA cloning. The primary sequence of the protein was deduced from the cDNA clones, with the sequence being found homologous to various members of the immunoglobulin protein super-family, especially to those molecules involved in cell adhesion. Schofield et al., *The EMBO Journal*, *8*:2 (1989) pp. 489-495.

Summary of the Invention

In one aspect of the present invention, a DNA segment corresponds to newly discovered clones. This DNA segment is prepared so as to be in the antisense direction. The product of DNA construct hybridizes with endogenous mRNA for an opioid binding protein, and is useful within a cell (cultured or in a transgenic animal) in reducing sensitivity to narcotic analgesics. Oligonucleotide constructs of the invention can also be RNA. Whether the oligonucleotide constructs are RNA or DNA, they preferably are modified to resist degradation *in vivo*. Uses of the inventive oligonucleotides include as diagnostic or therapeutic agents or as probes. As therapeutic agents they preferably include a means for therapeutically administrating to a patient. Sequences as small as about 12 bases (when single stranded) or about 12 base pairs (when double stranded) can be used, such as in therapeutic treatments of narcotics addicts.

Brief Description of the Drawings

Figure 1 graphically represents the antinociceptive activity of morphine sulfate in the presence of naloxone after control animals (non-transgenic) and inventive animals (transgenic) had received morphine pretreatment, with the inventive transgenic animals showing a marked reduced sensitivity to morphine; and,

Figure 2 graphically represents the cDNA of an inventive gene in which the coding region and the region used for antisense generation is shown (where most of the 5' region shown as "antisense fragment" was ligated into the mammalian expression vector pSVL SV40 and microinjected into mouse zygotes to prepare the transgenic animals to which the Fig. 1 data relates).

Detailed Description of the Preferred Embodiments

The present invention relates to sensitivity (such as by a cell or an animal) to narcotic analgesics. By "narcotic analgesic" is meant the various alkaloids of opium such as morphine, morphine salts (such as morphine hydrobromide, morphine hydrochloride, morphine muscate, morphine oleate, morphine N-oxide, and morphine sulfate), and morphine analogs and salts such as normorphine, diacetyldihydromorphine, diacetylmorphine hydrochloride, codeine and diacetylmorphine (heroin). Other widely used narcotic analgesics include alphaprodine, methadone, merperidine, levorphanol, propoxyphene, fentanyl, oxymorphone, anileridine and metopon. Also included as narcotic analgesics are the endogenous opioids (e.g. the endorphins) and the synthetic opioid peptides (e.g. D-Ala-2, D-Leu-5 enkephalin).

As is well known, continued use of at least many of th se narcotic analgesics leads to habituation or addiction. However, despite th ir abuse potential, thes narcotic analgesics have therapeutic uses, for

example with patients requiring chronic treatment to ease pain.

Even in such therapeutic uses, though, patients typically develop increasing tolerances to these narcotic analgesics, so that increasingly potent doses are required to achieve relief from pain. Undesired side effects then tend to develop to large, chronic doses of the narcotic analgesics.

The agonistic actions and dependence-producing properties of narcotic analgesics can be, and are, studied in various mammalian species besides humans, since practical and governmental considerations frequently require that studies be first done in small rodents and/or monkeys before the analgesic properties of pharmaceuticals are tested with humans. To the present, however, all drugs that have morphine-like properties in mammals other than man have been found to be morphine-like in man, and a variety of analgesic assays have been developed with animals which have gained widespread acceptance for predicting properties in humans.

The present invention generally relates to several clones pertaining to opioid receptor functions. A particularly preferred, novel clone of this invention (sometimes hereinafter called "*DUZ*1", whose sequence is given by SEQ ID NO:1) codes for a protein with opioid receptor function. We have made mice transgenic to a unique region of anti-sense *DUZ*1 and have demonstrated that these transgenic animals exhibit reduced sensitivity to the antinociception effects of morphine. These transgenic animals, and the underlying oligonucleotide constructs themselves, are useful, for example, in diagnoses of and treatments for addiction to narcotic analgesics.

For example, applications of the invention include therapeutic methods in treating addicts who can

potentially be cured of their addiction (because the invention can be used in ways that do not lead to tolerance development). In addition, patients requiring chronic treatment with narcotics to ease pain, such as terminal cancer patients, can be treated in accordance with the invention by administrations of antisense fragments to block the expression of the opioid receptor gene. This means the patient would still have the pain killing benefit of the narcotic analgesic, such as morphine, but that development of tolerance would be blocked. As a consequence, lower doses of the narcotic analgesic could be used and the various, known side effects (such as respiratory depression and constipation), which result form chronic treatment with high dosages of narcotics could be lessened.

Diagnostic kits in accordance with aspects of the invention can be used to determine if a patient is addicted and/or the degree of addiction. For example, cell samples taken from the patient can be screened with oligonucleotides of the invention. Detection of the opioid receptor gene in cells of a patient, such as, for example, blood cells, permits the screening for persons with tolerance to narcotic analgesics (that is, addicts) and differentiates from persons with a narcotic analgesic naivety.

When the invention is used therapeutically by administrating to patients, then single stranded forms tend to be more efficacious; however, double stranded forms tend to be more resistant *in vivo* (that is, to enzyme degradations and the like), although the action tends to be not as rapid as when singled stranded forms are used.

The inventive oligonucleotides can also be used as probes for discovering human genomic sequences and for discovering other subunits of opiate receptors.

The sequences we have given by SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3 are cDNA. When one wishes to use oligonucleotides of the invention but of a RNA form, then any of the well-known procedures for preparing oligonucleotides with RNA nucleotides (e.g. where the thymines are replaced by uracils) may be utilized.

DNA or RNA constructs of the invention may have at least a portion of the normal phosphodiester backbone modified in order to limit degradation *in vivo* or to convey resistance to protease or other enzymes. This is because even though antisense oligonucleotides have been shown to act as specific inhibitors of gene expression in a variety of *in vitro* systems, one of the major problems encountered is the rapid degradation in cells or culture media of the naturally occurring phosphodiester backbone (see, for example, Hoke et al., *Nucl. Acids Res., 19* (1991), pp. 5743-5748. Methods to replace all or part of the sugar-phosphate backbone of a stretch of DNA and of RNA are being reported, such as by Nielsen et al., *Science, 254* (1991) pp. 1497-1500. These chimeric oligonucleotides can form stable heteroduplexes with complementary single-stranded DNA.

Constructs of the invention thus usefully can have all or a portion of the normal phosphodiester backbone replaced with a variety of synthetic or semi-synthetic analogues capable of mimicking the distance and orientation of the native nucleobase substituents. Such modifications may be by means of a methylphosphonate, a phosoramidate, a phosphorothioate, a 2'-o-alkyl, a polyamide (2-aminoethylgylcine) or a modification of ribose to a cycloalkane or partially unsaturated cycloalkane.

Portions of clones described in an 1989 articl from work conducted in our laboratory (Schofield et al., *supra*) were used to prepare antisense cDNA. A

plasmid was then constructed containing the insert of rat OBCAM cDNA using the eukaryotic expression vector, pSVL. As illustrated by Example 1, when cells used as a model system containing a homogenous population of δ-opioid receptor were transfected with the plasmid, then opioid binding was blocked.

Clones of the invention have some coding regions common to the previously published work of Schofield et al., *supra*, but the inventive clones have unique regions. These unique regions are preferred in preparing the probes, DNA or RNA constructs, the therapeutic and/or diagnostic agents, and in providing transgenic animals with an altered response to narcotic analgesics.

Example 2 describes our isolation of three clones. The largest clone we sometimes call "*DUZ*1" (the sequence of which is given by SEQ ID NO:1) with a reading frame comparable to OBCAM in its C-terminal 318 amino acids, but differing in its N-terminal amino acids, differing in all of its 5'-noncoding region, and differing in some of the 3'-noncoding region.

Most of this unique 5'-noncoding region was then used as described by Example 3 (in the antisense orientation) in raising transgenic mice having a dramatically altered response to morphine (taking about three times as much morphine to kill pain as with control animals). These transgenic animals apparently do not develop tolerance to morphine.

The clone we sometimes call "*SG*13" (the sequence of which is given by SEQ ID NO:2) and the third clone we sometimes call "*SG*8" (the sequence of which is given by SEQ ID NO:3) are also useful in antisense constructs and applications. The SEQ ID NO:3 clone also has a unique 5'-noncoding region.

The inventive transgenic animals, for example, can also be used to provide cell cultures where somatic

cells from the animals are cultured, and then can be used in a variety of diagnostic techniques and assays.

Transgenes introduced into animals in order to make transgenic, non-human animals of the invention contain an oligonucleotide sequence introduced into the animals, or an ancestor of the animal, at an embryonic stage. The oligonucleotide sequence is expressible in the animals and is effective to alter the animal's response to a narcotic analgesic, as will be exemplified by Example 3. The oligonucleotide sequence introduced into the animals forms a transcriptional unit expressible in the animals as a messenger RNA compound capable of hybridizing to endogenous mRNA for an opioid binding protein. An altered response by the animal to a narcotic analgesic is, for example, a reduced sensitivity to narcotic analgesic (e.g., such as morphine, morphine analogues, endogenous opioids, and synthetic opioid peptides). For example, the oligonucleotide sequence introduced into the animals is preferably selected from all or part of the sequences illustrated by SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, but wherein the sequence selected (or its portion) is read in the antisense direction. Thus, Example 3 illustrates such a use where the first 500 base pairs of SEQ ID NO:1 (a novel portion of SEQ ID NO:1) was used, but used in the antisense direction in constructing the transgene.

Thus, aspect of the invention is the use of DNA or RNA constructs comprising a segment corresponding to all or part of SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, but reversed with respect to the direction shown (and having RNA bases, if the construct is RNA). The segment preferably is sufficient in size to hybridize with endogenous mRNA for an opioid binding protein. We believe a sufficient size is as short as 12 bases (when single stranded) or 12 base pairs (when double

stranded). Shorter sequences than this lose specificities.

The inventive constructs may be operatively linked to an expression vector, such as a mammalian expression vector or a retroviral expression vector, or not. That is, in many therapeutic applications an expression vector is believed not necessary, and the construct can be, for example, directly administered (in single or double stranded form) to a patient by a variety of administration means. However, as earlier mentioned, constructs of the invention preferably have been modified for resistance to degradation, such as where at least a portion of the normal phosphodiester backbone is modified with an analogue that is effective to limit degradation *in vivo*.

We believe that therapeutic uses of our DNA constructs include administration to patients who are addicted to narcotic analgesics in order to assist these patients in curing their addiction while avoiding development of tolerance. For example, the smaller inventive antisense constructs can be used to suppress expression of the gene.

We anticipate administration in amounts of 10 nmol or greater will be useful with the actual dosages and the duration of therapy to be determined by th treating physician and in accordance with developing oligonucleotide therapies. We believe a variety of administration means and methods will be useful, including i.v., transdermal, transnasal, and patch techniques, as well as microinjection. Thus, the invention includes diagnostic or therapeutic agents comprising an oligonucleotide with at least about 12 bases when single stranded and about 12 base pairs when double stranded and having a backbone, the oligonucleotide substantially corresponding to DNA bases (or two RNA analogous thereto) of all or a portion of SEQ ID

NO:1, SEQ ID NO:2, and SEQ ID NO:3, but in the antisens direction thereof, and including a means for therapeutically administrating the oligonucleotide to a patient. Such therapeutic administrating means includes patches and physiologically acceptable solutions and carriers.

Aspects of the invention will now be illustrated by Examples 1-3 and with reference to Figs. 1 and 2, which are meant to illustrate but not limit the invention.

Abbreviations used are: OBCAM, opioid-binding cell adhesion molecule; cDNA OBCAM, DNA copy of gene encoding OBCAM; bOBCAM, bovine gene encoding opioid-binding cell adhesion molecule; rOBCAM, rat OBCAM; pBOM, cDNA for bovine opioid-binding molecule; pROM, cDNA for rat opioid-binding molecule; bp, base pairs; PCR, polymerase chain reaction; RT, reverse transcriptions; HEPES, 4-(2-hydroxy)ethyl-1-piperazineethanesulfonic acid; aa, amino acid(s); *DUZ2*, clone 2 from UZ library; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); UZ, cDNA library created using rat brain poly(A)$^+$ RNA (Clontech) and Stratagene's Uni-Zap XR.

EXAMPLE 1

Plasmid Construction for Cell Line Transfection

Plasmids were constructed using standard recombinant techniques. The eukaryotic expression vector, pSVL was used. The rat OBCAM cDNA was digested with *Sma*I, and a 649-bp fragment was isolated on agarose gel. This *Sma*I fragment bracketed the AUG codon of rat OBCAM cDNA, containing 272 bp upstream of the AUG and 377 bp downstream. This fragment was ligated to a *Sma*I-cleaved preparation of pSVL, followed by transformation of *Escherichia coli* DH5α competent cells. Plasmid DNAs from twenty of these ampicillin-resistant colonies were isolated and nine out of twenty colonies were shown to

contain the 649-bp insert. The orientation of the insert was verified by the cleavage of internal *XhoI* site. Two colonies which carried either sense or antisense orientation were selected and named "pSVL sense" or "pSVL antisense", respectively. To ensure that the orientations of pSVL sense and pSVL antisense were correct, the plasmid DNAs were digested by th enzymes *KpnI* and *PvuII*; the products corresponded in size to those expected from pROM sequences.

Cell Culture and DNA Transfection

NG108-15 neuroblastoma x glioma cells were grown in tissue cultures. Tissue culture medium components were purchased from GIBCO. Fetal calf serum was obtained through Hyclone.

Calcium phosphate-mediated DNA transfection was carried out as follows. On day 1, cells were plated at a density of approximately 1.2 x 10^6 cells/10 cm dish. On day 2, 3 hours before transfection, all the medium was removed and replaced with 10 ml of fresh medium. Twenty to 30 μg of plasmid DNA (in the ratio 1:50 of pSVL sense or pSVL antisense plus selectable plasmid pSVNeo) were transfected/plate. The plate was incubated overnight at 37°C in a 5% CO_2 atmosphere. On day 3, the medium was removed, and cells were washed twice with phosphate-buffered saline, 20 ml of fresh medium was added, and incubation was shifted to a 10% CO_2 atmosphere at 37°C overnight. On day 4, the cells were divided into an appropriate ratio (1:3 to 1:5) and incubated in fresh medium in a 10% CO_2 atmosphere, 37°C for 24 hours before selection was started. On day 5, the medium was removed, and the selection medium (containing G418 sulfate, actual concentration 400 μg/ml) was used for the next 4 weeks. The medium was changed twice/week, until resistant colonies were visualized. Generally speaking, in each 10-cm dish 10-

30 colonies were observed, and a single colony was isolated and expanded to an individual cell line.

Nucleic Acid Preparation

Poly(A)$^+$ RNA from these clones was isolated from cultures in 10 cm dishes. Poly(A)$^+$ RNA of rat brain was purchased from CLONTECH. Genomic DNA was isolated from rat brain, NG108-15, ST8-4, and ST7-3 cells.

Polymerase Chain Reaction

Oligodeoxynucleotide primers (synthesized by Northern Biosciences Incorporated, Hamel, MN) were prepared (with numbers in brackets indicating the positions in the sequence of pROM):

RPJ1, [nucleotides 482-499];
RPJ2, [nucleotides 620-603];
RPJ3, [nucleotides 158-172];
RPJ4, [nucleotides 760-743];

mRNA PCR

Reverse Transcription. Ten ng of poly(A)$^+$ RNA were added in 20 μl of reaction solution containing 50 mM Tris-HCL (pH 8.3), 3 mM $MgCl_2$, 75 mM KCl, 2.5 mM dithiothreitol, 1 mM of each dNTP, 20 units of RNase inhibitor (Promega Biotec), 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO/Bethesda Research Laboratories), and 50 pmol of random hexamer, and incubated for 1 hour at 42°C. The reaction was stopped by heating at 99°C for 5 minutes.

Amplification. To 20 μl of mRNA/cDNA hybrid, 80 μl of PCR solution containing 15 pmol of each primer and 2.5 units of Taq DNA Polymerase were added, and the mixtur was overlaid with 60 μl of mineral oil (Perkin-Elmer Cetus). Final concentration of $MgCl_2$ was 3.8 mM. After the reaction mixture was denatured by heating at

94°C for 5 minutes, cDNA was amplified in a Perkin-Elmer DNA thermal cycler for 35 cycles, with a denaturation step at 94°C for 30 seconds, an annealing step at 55°C for 30 seconds, and an extension step at 72°C for 30 seconds.

DNA PCR

DNA PCR was performed in 100 µl containing 50 mg of genomic DNA, 15 pmol of each primer, 20 nmol of each dNTP in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.001% (w/v) gelatin. The thermal cycling condition was as described above.

These PCR products were electrophoresed on the agarose gel and transferred to the MagnaGraph nylon membranes. The membranes were subjected to hybridization using ^{32}P-labeled *Sma*I fragment.

Northern Blot Analysis

Seven µg of poly(A)$^+$ RNA were size-fractionated on a denaturing formaldehyde/agarose (1%) gel and transferred to a MagnaGraph nylon membrane (Micron Separations Inc.). The *Sma*I fragment of pROM and 138 bp of PCR product between RPJ1 and RPJ2 were labeled using [α-^{32}P]dCTP and random hexamers (Feinberg and Vogelstein, 1983). Hybridization was carried out as described previously (Thomas, 1980), using ^{32}P-labeled probes. The nylon membrane was washed with 40 minutes incubations at 68°C in 1 x SSC and 0.5% (w/v) sodium dodecyl sulfate (Ann et al., 1987). The SSC composition had 0.5 M sodium chloride and 45 mM sodium citrate.

Sequencing of PCR Product

PCR products between RPJ1 and RPJ2 were extracted from the reaction solution. For making blunt ends, the extracted DNA was incubated with 2 units of T4 DNA polymerase (Promega) in the presence of 0.1 mg/ml of

bovine serum albumin and 100 μM of each dNTP at 37°C for 5 minutes. The reaction was stopped by heating at 75°C for 10 minutes. The cDNA containing blunt ends was collected by using spin column (Boehringer Mannheim) and cloned into the *Sma*I site of pUC19 vector (GIBCO/BRL). Supercoiled plasmid DNA was sequenced with Sequenase (United States Biochemical).

Membrane Preparation

Cells were harvested in phosphate-buffered saline/EDTA (phosphate buffer + 1 mM EDTA), homogenized in 25 mM HEPES (pH 7.4), 0.32 M Sucrose, and centrifuged at 1,000 x *g*. The resulting supernatant was centrifuged at 100,000 x *g*, and the pellet resuspended in 25 mM HEPES (pH 7.4).

Receptor Binding Assay

Cell membranes (about 300 μg) were incubated for 90 minutes at room temperature with labeled ligand (2 nM unless otherwise noted) in the presence or absence of 1 μM unlabeled ligands, in 25 mM HEPES (pH 7.4). Th ligands used for the binding assays were tritiated diprenorphine (opioid), scopolamine (muscarinic), rauwolscine (α_2-adrenergic), and ^{125}I-insulin. The unlabeled ligands used for opioid, muscarinic, α_2-adrenergic, and insulin binding assays were D-Ala^2-D-Leu^5-enkephalin (DADLE) or various other opioid ligands, atropine, phentolamine, and insulin, respectively. The reaction was terminated by filtration on GF/B filters (Whatman), the filters washed three times with the HEPES buffer, and allowed to stand with Scintiverse-BD (Fisher) overnight before counting in a scintillation count r.

Results

Use of RT-PCR for the Detection of OBCAM cDNA in NG108-15 Cells. Before transfecting NG108-15 cells with sense and antisense cDNA derived from pROM, it was essential to demonstrate that the pROM sequence, or a highly similar homolog, was present in these cells. Northern blot analysis, using pROM as probe, indicated that transcripts for OBCAM were barely detectable in 7 μg of poly(A)$^+$ RNA prepared from NG108-15 cells (data not shown). This observation was unexpected in light of the fact that NG108-15 cells are derived from rat and mouse cell lines and would thus be expected to contain either pROM itself, from rat, or a highly homologous molecule that is presumably present in mouse.

Northern blot is a relatively insensitive assay system. As a more sensitive approach, we used RT-PCR. Two pairs of primers were designed and synthesized from this experiment. RPJ1 and 2 consisted of pROM nucleotides 682-499 and 603-620, respectively, and RPS5 and 6 contained pROM nucleotides 158-172 and 760-743, respectively. The latter two were chosen to span most of the region used for sense and antisense cDNA; however, this region contained some non-coding sequence, which might be expected to be of lower homology between different species. Thus, we also used RPJ1 and 2, which spanned a short region, completely within the reading frame.

Products using RPS5 and 6 of the expected size of approximately 600 bp were detected using sense- and anti-sense-transfected cells, but not non-transfected cells. Using RPJ1 and 2, however, the expected product of about 140 bp was detected in both control and transfected cells.

Two clones, NGJ2 and NGJ6, from PCR products between RPJ1 and RPJ2 were sequenced. These sequences diff red in only a few nucleotides from pROM or pBOM,

and these differences in nucleotide sequence did not affect the derived amino acid sequences, which were identical to bovine and rat OBCAM. The slight differences between the two PCR-derived clones, on the other hand, might have resulted from either heterogeneity in the NG108-15 cells or from errors introduced by the Taq polymerase. This conservation in the derived amino acid sequences suggested that these RT-PCR products were the corresponding cDNAs of NG108-15 cells.

Identification of Clones Successfully Transfected with Sense and Antisense DNA. To study the function of OBCAM further, we have attempted to generate stably transfected sense and antisense pSVL pROM in NG108-15 cells. Twelve individual clones from pSVL sense and nine clones from pSVL antisense transfection were selected on the basis of G418 resistance and expanded into 10 cm culture dishes. Clones containing the appropriate RNA could be identified by Northern blot analysis using the *Sma*I fragment as probe. However, 7 μg of poly(A)$^+$ RNA from NG108-15 cells not transfected with rat OBCAM DNA were not hybridized with ^{32}P-labeled *Sma*I fragment of pROM.

Determination of the Copy Number of pROM Inserts in the Two Transfected Cell Lines. Genomic DNA was isolated from NG108-15, ST8-4, and ST7-3 cell lines, and the pROM inserts were amplified by PCR an analyzed on 2% agarose gel. An estimate of the number of ST7-3 antisense and ST8-4 sense inserts was obtained by using densitometry scanning to compare the number of antisense sequences in known amounts of clone pSVL antisense DNA to antisense or sense sequence present in 50 ng of either ST7-3 or ST8-4 genomic DNA. An estimate of the copy number was achieved by comparing the relative intensity of PCR amplification products from ST7-3 and ST8-4 cells to the intensities of the identically sized PCR products of a known copy number of pSVL antisense

DNA. This analysis indicated the presence of approximately 12 copies of pSVL antisense in ST7-3 genome and four copies pSVL sense in ST8-4 genomes.

Receptor Binding of Opioids and Other Ligands to Transfected Cell Lines. The OBCAM-transfected cell lines ST8-4 (sense) and ST7-3 (antisense) were tested for the presence of receptors for opioids as well as for several other classes of ligands. Binding of the opioid [^{3}H] diprenorphine was reduced by 80% in the antisense-transfected cells, as compared to non-transfected cells. [^{3}H] Diprenorphine binding to sense-transfected cells was reduced about 10%, which was statistically nonsignificant. In contrast, binding of the α_2-adrenergic ligand [^{3}H]rauwolscine, the muscarinic ligand ^{3}H scopolamine, and ^{125}I-insulin were not affected by antisense (or sense) transfection.

The properties of the opioid receptors in the sense- and antisense-transfected cell lines were further tested. Scatchard analysis indicated that essentially all of the 80% reduction of [^{3}H] diprenorphine binding in the antisense-transfected cells was due to a decrease in B_{max}, while K_d was unaffected. The opioid receptors in both cell lines retained the δ characteristics of non-transfected NG108-15 cells, as indicated by the rank order of affinity of various opioid ligands: DADLE > ß-endorphin > morphine > U-50,488H. In addition, the opioid receptors present in either transfected cell retained their stereoselectivity.

Finally, the effect of chronic opioid agonist treatment on the transfected cell lines was examined. Incubation of sense-transfected cells with 100 nM DADLE for 24 hours induced a reduction of 80% of opioid receptors, essentially the same as that observed in non-transfected cells. However, the same treatment did not further reduce the opioid receptor binding of antisense-transfected cells. These experiments show that cells

transfected with antisense cDNA to OBCAM can block opioid binding.

EXAMPLE 2

This example illustrates our having conducted a search for cDNAs coding for an OBCAM-like protein, but with one or more additional regions (such as regions coding for a transmembrane and/or intracellular domain, which domains are missing in OBCAM). We found a clone about 3.0 kb in length that was highly homologous to bOBCAM throughout the latter's open reading frame, but with unique regions.

General Approach. Two cDNA libraries were screened, one purchased from Stratagene (SG; amplified one time), and one created in our laboratory using rat brain poly(A)$^+$ RNA (Clontech) and Stratagene's Uni-Zap XR (UZ). The library was screened with probes representing two different regions of OBCAM, one from the bovine clone (bOBCAM) and one from the partial rat clone (rOBCAM). Comparison of these two clones revealed that the coding region was highly conserved, so both probes were generated from within this region.

Screening of the UZ Library. Screening of the UZ library (of which 1/5 was packaged and plated) produced only two clones, *DUZ*1 (SEQ ID NO:1) and *DUZ*2, both about 3.0 kb in length. From terminal sequencing of the 5' and 3' ends, in addition to restriction maps, it was concluded that they were identical. Hence, only *DUZ*1 (SEQ ID NO:1) was subjected to complete sequencing. The resulting sequence was highly homologous to bOBCAM throughout most of the latter's open reading frame but had no homology in a portion of the 5'-noncoding region. *DUZ*1 (SEQ ID NO:1) contained a slightly shorter putative reading frame, of 338 aa; the C-terminal 318 aa were

virtually id ntical to those of OBCAM, including the same stop codon, but the N-terminal 20 aa were unique.

Screening of the SG Library. Using high stringency conditions, 18 clones were obtained from the SG library, ranging in size from 0.6 kb to 3.5 kb. Only those clones greater than 1.0 kb were further characterized, and terminal sequencing quickly identified which clones most likely contained an entire coding sequence. Most of these clones appeared to be identical or highly homologous with *DUZ*1 (SEQ ID NO:1) in the 3' untranslated region and the 3' portion of the coding region. These clones contained a stop codon in the same position as found in bOBCAM. However, two of these clones, *SG*8 (SEQ ID NO:3) and *SG*13 (SEQ ID NO:2), proved to be of particular interest.

*SG*13 (SEQ ID NO:2) was identical to rOBCAM in the area where the two clones overlapped; however, unlike rOBCAM, *SG*13 (SEQ ID NO:2) contained a complete reading frame, a short 3' non-coding region, and a 5' non-coding region that extended upstream further than that of rOBCAM. We believe that *SG*13 (SEQ ID NO:2) represents a sequence extended with respect to rOBCAM, and which contains an additional unique 5' region.

*SG*8 (SEQ ID NO:3) also had a complete reading frame, identical to that of *SG*13 (SEQ ID NO:2), and was also identical to the latter throughout its somewhat shorter 3' non-coding region and in the 200 or so nucleotides immediately upstream of the putative start codon. However, the 5' non-coding region further upstream had no significant sequence homology with *SG*13 (SEQ ID NO:2), suggesting that it may involve a region regulating translation.

Physiological Significance of Clones. Both *DUZ*1 (SEQ ID NO:1) and *SG*13 (SEQ ID NO:2) have been found to be located on mouse chromosome 9. Preliminary *in situ* hybridization studies, using unique regions of

these two clones, reveal that their cDNAs are distributed differently in different brain regions, suggesting they are under different transcriptional regulation. One or more of the unique regions in these new clones may be involved in negatively regulating gene expression.

EXAMPLE 3

Transgenic mice containing antisense oriented region from *DUZ1* (SEQ ID NO:1) (prepared in a double-stranded construct) exhibited reduced sensitivity to the antinociceptive effects of morphine. The transgene used in these studies was the first 500 base pairs as a fragment of the clone *DUZ1* (SEQ ID NO:1), but constructed to be in the antisense orientation.

Transgenic mice (founders) were identified and non-transgenic littermates were used as controls for antinociceptive tests. Of 33 *DUZ1* (SEQ ID NO:1) founders, 18 (54%) contained the transgene. One of the transgenic males (#1355) was mated with females of an inbred strain (C57BL/10) to produce N1 generation mice; of 29 offspring, 8 (28%) contained the transgene. Six transgenic (two males, four females) and six non-transgenic littermates (two males, four females) of similar age were grouped for antinociceptive tests.

Control mice, lacking the transgene, had an AD_{50} for morphine as shown in Table 1. These values were similar whether non-transgenic littermates of transgenic animals or age- and sex-matched naive C57BL/10 mice were used as a control group, as illustrated by the data of Table 1.

TABLE 1

Antinociceptive Activity (AD_{50}) of Morphine Sulfate in Transgenic Mice

	Nontransgenic	Transgenic
Naive	7.05 ± 1.18	---
Founders	7.07 ± 1.54	11.22 ± 1.32 $p < 0.02$
N1	7.07 ± 1.18	22.44 ± 1.22 $p < 0.001$

Fertilized hybrid zygotes were obtained approximately 12 hours postcoitus from the mating of (C57BL/6 female x SJL male) F2 hybrid mice. Zygote isolation, injection, and reimplantation into the oviducts of pseudopregnant Swiss-Webster recipient females. Transgenic offspring were identified by using the polymerase chain reaction on mouse tail DNA samples. Approximately 1 cm of tail was removed and incubated overnight at 55°C in 200 µl of 50 mM Tris, pH 8, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, and 2.5 mg/ml proteinase K. The digested solution was precipitated with isopropanol, spooled, transferred into 50 µl water, and boiled for 15 minutes.

Antinociceptive activity of morphine sulphate was determined by the tail flick method. Morphine sulphate was injected s.c. Because of the small number of animals available (6 in each group), the up-and-down method was used to determine AD_{50}. (Dixon, *Amer. Statistical J.*, (1965), 967-978.)

Manipulation of zygotes had no effect on morphine sensitivity of the animals as non-transgenic adults. In contrast, founder mice, and first generation containing the antisense construct, had a morphine AD_{50} of 11.22±1.32, while the N1 generation mice, the second generation, had a morphine AD_{50} of 22.4±1.22. Thus the presence of the transgene in mice was correlated with a

reduced sensitivity to morphine, and this reduction in sensitivity was increased in the next generation. N1 generation mice derived from founder male 1355 contained the antisense transgene in another inbred strain (DBA/2J) show significantly less sensitivity to morphine. This observation, taken with the difference in sensitivity to morphine in founders and C57BL/10 N1 offspring suggests the expression of the transgene is influenced by the genetic background of the mouse strain harboring it.

In addition, N1 generation of transgenic and non-transgenic animals were tested for acute tolerance. Animals were first assayed for tail-flick latency in the absence of any drug, to generate baseline data. Thirty minutes later, the mice were pretreated with a fixed dose of morphine given s.c. in 0.1 ml physiological saline (see below), and tail flick latencies monitored again until they had returned to baseline. Morphine antinociception was then determined in the presence of 125 μg/kg s.c. naloxone, given ipsilateral to the morphine sulfate administration at the same time. As in the initial antinociceptive tests, the morphine AD_{50}s were determined by the up-and-down method.

Four sets of acute tolerance tests were carried out, employing morphine pretreatment doses of 0, 10, 30 and 100 mg/kg. Because of the small number of transgenic mice available, the same animals were used for these studies as for the initial tests of antinociception. To be certain there were no residual drug effects from the earlier tests, the first acute tolerance studies were begun two weeks after the initial antinociceptive tests, and each subsequent study (employing a higher pretreatment dose of morphine) was carried out two weeks after the preceding one. Finally, two weeks after the last acute tolerance study, the morphine AD_{50}s of animals were again determined (no

naloxon), to confirm that no changes in this parameter had occurred over the course of the experiments.

As shown in Figure 1, the AD_{50} of morphine was increased in the acute tolerance studies, and the higher the pretreatment dose of morphine, the higher the AD_{50}. With a maximum pretreatment dose of 100 mg/kg, the AD_{50} in the nontransgenic animals reached a maximum of 67.45 mg/kg, a nearly 10-fold increase over the control mice not pretreated with morphine. In contrast, the AD_{50} of morphine in the transgenic animals (N1 generation) increased relatively slightly with morphine pretreatment dose, with a maximum value of 35.53 mg/kg in transgenic animals pretreated with 100 mg/kg morphine. Since the control AD_{50} in these animals was elevated to 22.44 mg/kg, this represented an increase of only about 50%.

By this test, too, mice containing th transgene showed a reduced sensitivity to morphine. In this case, this reduced sensitivity was manifested in less of an effect of morphine pretreatment in raising the AD_{50}.

These data show that we have successfully transfected mice with antisense cDNA coding for a portion of an opioid binding protein. The presence of this cDNA results in reduced sensitivity to morphine, by two different tests. The line of transfected animals should be useful in a wide variety of applications relating to opioid function.

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the inv ntion, which is defined by the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lee, Nancy M.
Loh, Horace H.
Lippman, David

(ii) TITLE OF INVENTION: Oligonucleotide Sequences and Transgenic Animals Transfected Therewith Having Reduced Sensitivity to Narcotic Analgesics

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: J. Suzanne Siebert
(B) STREET: Four Embarcadero Center
Suite 1450
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94111

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0,
Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: 10-APR-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Siebert, J. Suzanne
(B) REGISTRATION NUMBER: 28,758
(C) REFERENCE/DOCKET NUMBER: 2983.1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415) 362-5556

(B) TELEFAX: (415) 362-5418
(C) TELEX: 278638 MGPS

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3069 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: YES
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rattus rattus
(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: brain
(vii) IMMEDIATE SOURCE:
(A) LIBRARY: library of Lee, Loh, Lippman (genebank accession #M88709)
(B) CLONE: clone DUZ1
(viii) POSITION IN GENOME:
(B) MAP POSITION: on mouse chromosome 9
(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGGGGCGAAG CTGGCTGCAT CCTGGGCTGG AGACCAGGAA GGCGGAATGA TAAGGCTTTG 60

GCATGGGTAT TAGTTAATTT CTGCCAGGAA GCAGCCTTTT CCTGTTCCTT GTGTCCTGAG 120

ACCTGCTAGG GTAAGATTTA GGGAAGGGCA TTAGACCCCT CCCGCTGCCC ACCCAGGAGC 180

GGGTGTGCCC ATGCGAGTCA CACTGGCATA CTCACACAAA TCCCAGCAGT
ACTGCTGCGC 240

GGTGAGGGGG TGCGCAAGGG ACAGGCTTCA ACTCCCCTTG GAGTGGAGCC
TTCCCAACAG 300

GAAAGTCTCC CGGTGTCGCT CAGGAGAGTG GAGCCAATGT GTGGCTTGGA
GGTGGTCTGC 360

TGTGCTCCCA GTGAAGATTG GTGCTGATGA TTCTTACCAG TTGTGTGTAT
GTGCAAGGGG 420

GAAAAAATCA GAGACGCTGT CTGCCTGCTC CCATCTCGCG CGCGCTCTCT
CTCTTCTGCT 480

CTCTCCCTCC CTTTGCAAAC ATTGGATTTA AACCTGCTCA GAATTCAGTA
CAGAGGAAGC 540

AGCCTCGGTG GTAGCAGCAG CAGCACCAGC AGCAGCAGCA CCAGCAGCAC
CAGCACCAGC 600

AGGAGCTCGC CGGGCCGCCG CGCACCACAG CCTCGAGATG TACCATCCCG
CCTACTGGAT 660

CGTCTTCTCG GCCACCACTG CCCTGCTCTT CATCCCAGGA GTGCCGGTGC
GCAGCGGAGA 720

TGCCACCTTT CCCAAAGCTA TGGACAACGT GACGGTCCGG CAGGGAGAGA
GCGCCACCCT 780

CAGGTGTACC ATAGATGACC GGGTCACCAG AGTAGCCTGG CTAAACCGCA
GCACAATCCT 840

CTACGCTGGG AATGACAAGT GGTCCATAGA CCCTCGAGTG ATCATCTTGG
TCAACACGCC 900

TACCCAGTAC AGTATCATGA TCCAGAATGT GGATGTTTAT GACGAAGGTC
CGTACACCTG 960

CTCTGTGCAG ACAGACAATC ACCCCAAAAC CTCCCGGGTC CACCTCATAG
TGCAAGTTCC 1020

TCCCCAGATA ATGAACATCT CTTCAGACAT TACTGTGAAT GAGATAAGCA
GTGTGACCTT 1080

GTTATGTCTC GCAATTGGCA GACCAGAACC AACAGTGACA TGGCGACACC
TGTCAGTCAA 1140

GGAAGGCCAG GGCTTTGTGA GTGAAGATGA ATACCTGGAA ATCTCAGACA
TCAAACGCGA 1200

CCAATCTGGA GAGTATGAGT GCAGCGCCTT GAATGATGTC GCTGCACCTG
ATGTTCGGAA 1260

AGTAAAAATC ACTGTAAACT ATCCTCCCTA TATCTCAAAA GCGAAGAACA
CTGGCGTTTC 1320

AGTAGGCCAG AAGGGCATCC TGAGCTGTGA AGCCTCTGCT GTCCCCATGG
CTGAATTCCA 1380

GTGGTTCAAG GAAGATACCA GGTTAGCCAC TGGCCTGGAT GGCGTGAGAA
TTGAGAACAA 1440

AGGCCGCATA TCCACTTTGA CTTTCTTCAA TGTCTCAGAG AAGGATTATG
GGAACTATAC 1500

CTGTGTGGCC ACAAACAAGC TTGGGAACAC CAATGCCAGC ATCACCCTGT
ATGGGCCTGG 1560

AGCAGTCATT GATGGTGTAA ACTCGGCCTC TAGAGCACTG GCTTGTCTCT
GGCTCTCAGG 1620

GACCTTCTTT GCCCACTTCT TCATCAAGTT TTGATAAGAA ACCTTAGGTC
CTCTGAGCAT 1680

CGCCTGCTTC TCCATATCAC AGACTTTAAT CTACACTGCG GAGGGGCAAA
CCAGTTTGGG 1740

CTTTCTTTTG GTTATTTTTT TGTTCTTCTT GACTGTTTAG TTTTTTGGTT
TGATTTCTGG 1800

GATTTTCAAT TTGATTTGTT TTTCTTTTTT CGTTTGAATG GACCGGGGTT
GGGGGTTGGG 1860

ATGGGCAGGG TTCTACCACG AGTAGGATAA TCAGGTATTG GTGGGCCCCC
AAATGGAATA 1920

TATTCCTGCT ACCTTGGCCT TCCTTTTCTC TACTTCTCTT CCTACCACCA
TTAACACACA 1980

AACACACAAG CACACGCCCT AAAGATGGCC TAAAAAATGT CCCATGACAC
GCACCCTGAA 2040

GGTACAACTT GGCCCACAGT GCAGTACACA ATAAGAGTTG CATCTACATT
TCCTGTTTTC 2100

TTTGTCCTTT AAGTTTTCAA TAAGACAGTT TAAAAAGAGC ACATCCTTAT
CCCTATGTTT 2160

GTATCACCTA TCCCATTAAG CTGCACACCT TTTCTAAGAA ACTTTCTTAC
TACATCCTCA 2220

ATGTGCACAC ATGTACATTC TCATAAAAAT TTTACCATCT TCTCTGGCCA
TTCTGCTTAA 2280

TCTGCATTTT CCACTAGCAG TAAGACTTAC AGGCTTGATG AAATATACAT
GTATCCAAAG 2340

CTACAAATTT AGAAGTCACG AGGGAAGTCT ATCTAGGGGT AGTACTTCCC
TTAAAATGCT 2400

AATGCAACTC ATAAAAAAGT GATCAATAGC TAGCTAATTA TATCAAGCTA
TCAAAGCAAT 2460

CATATTATTA TCCAGTTACT CAATTGATTT CTCTCTCCAT CTCCTTCTAC
ATACCTATCT 2520

TTCTTATAAT CTATTTGTTC AATTTATCTA ACTACTCTTT TTCTCTTCCC
ACTGCACTAT 2580

CCATTCATCT CATTTAAATC TCTAATCATA TTGTGACTGT GCCCTTATAT
TGTGTCTCTG 2640

CACTACCACC AAGACATTGC CACCTTCAAA ATTGCCTAGC AACTTCTTAT
GTGAAAGCCA 2700

GTAATGTCTC GGGCTAACTA CCCAGAAGAA CAATTTCCTA TACCATGGAT
CCTTGGGAAT 2760

GCACTAACCC ATCCACCCAA ATTAGGCCTT GTGAACAGAT GGACCAAAGT
AGCAATCTAT 2820

GGATCAGTCA CTCATGATTT TCCGAGAGAC TGTTCCCTTA GCCACCTTCT
CAGGAGGCAG 2880

GTCAGCCCCG AAGAAGCCTT GATTATGCTG AATTTCTCCT TTAACAGCTG
GAAAATTAAG 2940

GTACCAACCC TGTGCTTCTC TCAGCCTTTC AAGAAAAGTA CATGTCAGGA
ACTTGGAGAA 3000

ACTTCTTCAT GGCTGGCTTT TATTAGCAGA AAGAACCTGA CCAAAAAAAA
AACCAAAAAA 3060

AAAAAAAAA

3069

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2337 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rattus rattus

(D) DEVELOPMENTAL STAGE: Adult

(F) TISSUE TYPE: brain

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: stratagene (genebank accession #M88711)

(B) CLONE: SG13

(viii) POSITION IN GENOME:

(B) MAP POSITION: on mouse chromosome 9

(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTTTTTTTA TTTTTTATTT TTTTTACGAC ATCTCCTTTC TGTCAGAGAC ACTTGCCTTC 60

TTTGGGTTAT AAACTTTTGA TGCCAGACCT CTGCAGAATC GCCCAACTGA ATCTTAAAGT 120

AGCTTCACCG AGAGAGGCAG AGAACGCACA ATCTGTGACC TTCGTCCCTG CTCCTTTTTT 180

GTGTTCTCCC TCCTCCCGGG CTCCCTCCGC CCCAGGGAGC GTTGAGAAAG
CTCTTTTTTG 240

GATGCAGGAG GGGGCATCTG GTTCTGCCAG GCTGGAAAGC TGAGGCTGGA
GCAGAGGAAG 300

ACTTATTAGA CTCCGGAGAG CCTGGACTCA GCCTTGCCTT CTCCCGCTCC
CAGCTCCTGG 360

TTTGCTCTCT GTGTGCTTTC GTCCCTCAAC ATTCCGGCTA TTCTGAGAGG
GCAGGGGACA 420

AGGACCGTGC AGCTGCAAGA GTTCTAGGAA GTTGTGGCTG TCGAGAATGG
GGGTCTGTGG 480

GTACCTGTTC CTGCCCTGGA AGTGCCTCGT GGTCGTGTCT CTGAGGCTGC
TGTTCCTTGT 540

ACCCACAGGA GTGCCGGTGC GCAGCGGAGA TGCCACCTTT CCCAAAGCTA
TGGACAACGT 600

GACGGTCCGG CAGGGAGAGA GCGCCACCCT CAGGTGTACC ATAGATGACC
GGGTCACCAG 660

AGTAGCCTGG CTAAACCGCA GCACAATCCT CTACGCTGGG AATGACAAGT
GGTCCATAGA 720

CCCTCGAGTG ATCATCTTGG TCAACACGCC TACCCAGTAC AGTATCATGA
TCCAGAATGT 780

GGATGTTTAT GACGAAGGTC CGTACACCTG CTCTGTGCAG ACAGACAATC
ACCCCAAAAC 840

CTCCCGGGTC CACCTCATAG TGCAAGTTCC TCCCCAGATA ATGAACATCT
CTTCAGACAT 900

TACTGTGAAT GAGATAAGCA GTGTGACCTT GTTATGTCTC GCAATTGGCA
GACCAGAACC 960

AACAGTGACA TGGCGACACC TGTCAGTCAA GGAAGGCCAG GGCTTTGTGA
GTGAAGATGA 1020

ATACCTGGAA ATCTCAGACA TCAAACGCGA CCAATCTGGA GAGTATGAGT
GCAGCGCCTT 1080

GAATGATGTC GCTGCACCTG ATGTTCGGAA AGTAAAAATC ACTGTAAACT
ATCCTCCCTA 1140

TATCTCAAAA GCGAAGAACA CTGGCGTTTC AGTAGGCCAG AAGGGCATCC
TGAGCTGTGA 1200

AGCCTCTGCT GTCCCCATGG CTGAATTCCA GTGGTTCAAG GAAGATACCA
GGTTAGCCAC 1260

TGGCCTGGAT GGCGTGAGAA TTGAGAACAA AGGCCGCATA TCCACTTTGA
CTTTCTTCAA 1320

TGTCTCAGAG AAGGATTATG GGAACTATAC CTGTGTGGCC ACAAACAAGC
TTGGGAACAC 1380

CAATGCCAGC ATCACCCTGT ATGGGCCTGG AGCAGTCATT GATGGTGTAA
ACTCGGCCTC 1440

TAGAGCACTG GCTTGTCTCT GGCTCTCAGG GACCTTCTTT GCCCACTTCT
TCATCAAGTT 1500

TTGATAAGAA ACCTTAGGTC CTCTGAGCAT CGCCTGCTTC TCCATATCAC
AGACTTTAAT 1560

CTACACTGCG GAGGGGCAAA CCAGTTTGGG CTTTCTTTTG GTTATTTTTT
TGTTCTTCTT 1620

GACTGTTTAG TTTTTTGGTT TGATTTCTGG GATTTTCAAT TTGATTTGTT
TTTCTTTTTT 1680

CGTTTGAATG GACCGGGGTT GGGGGTTGGG ATGGGCAGGG TTCTACCACG
AGTAGGATAA 1740

TCAGGTATTG GTGGGCCCCC AAATGGAATA TATTCCTGCT ACCTTGGCCT
TCCTTTTCTC 1800

TACTTCTCTT CCTACCACCA TTAACACACA AACACACAAG CACACGCCCT
AAAGATGGCC 1860

TAAAAAATGT CCCATGACAC GCACCCTGAA GGTACAACTT GGCCCACAGT
GCAGTACACA 1920

ATAAGAGTTG CATCTACATT TCCTGTTTTC TTTGTCCTTT AAGTTTTCAA
TAAGACAGTT 1980

TAAAAAGAGC ACATCCTTAT CCCTATGTTT GTATCACCTA TCCCATTAAG
CTGCACACCT 2040

TTTCTAAGAA ACTTTCTTAC TACATCCTCA ATGTGCACAC ATGTACATTC
TCATAAAAAT 2100

TTTACCATCT TCTCTGGCCA TTCTGCTTAA TCTGCATTTT CCACTAGCAG
TAAGACTTAC 2160

AGGCTTGATG AAATATACAT GTATCCAAAG CTACAAATTT AGAAGTCACG
AGGGAAGTCT 2220

ATCTAGGGGT AGTACTTCCC TTAAAATGCT AATGCAACTC ATAAAAAAGT
GATCAATAGC 2280

TAGCTAATTA TATCAAGCTA TCAAAGCAAT CATATTATTA TCCAGTTACT
CAATTGA 2337

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2179 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Rattus rattus

(D) DEVELOPMENTAL STAGE: Adult

(F) TISSUE TYPE: brain

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: stratagene (genebank accession #M88710)

(B) CLONE: SG8

(viii) POSITION IN GENOME:

(C) UNITS: bp

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGTGTGATCA ATGGTCTTAG AAGGCTTGTT AGACAGCTGT GGGGCTCAGT CTTCTCGCAG 60

AATGTTAACA GTGGGAGCAG GGGCTGTCTC TGACTCTGTT GCCTGCCGGC AGTGAGCAAT 120

GGTCTTAGAA GACTTATTAG ACTCCGGAGA GCCTGGACTC AGCCTTGCCT TCTCCCGCTC 180

CCAGCTCCTG GTTTGCTCTC TGTGTGCTTT CGTCCCTCAA CATTCCGGCT ATTCTGAGAG 240

GGCAGGGGAC AAGGACCGTG CAGCTGCAAG AGTTCTAGGA AGTTGTGGCT GTCGAGAATG 300

GGGGTCTGTG GGTACCTGTT CCTGCCCTGG AAGTGCCTCG TGGTCGTGTC
TCTGAGGCTG 360

CTGTTCCTTG TACCCACAGG AGTGCCGGTG CGCAGCGGAG ATGCCACCTT
TCCCAAAGCT 420

ATGGACAACG TGACGGTCCG GCAGGGAGAG AGCGCCACCC TCAGGTGTAC
CATAGATGAC 480

CGGGTCACCA GAGTAGCCTG GCTAAACCGC AGCACAATCC TCTACGCTGG
GAATGACAAG 540

TGGTCCATAG ACCCTCGAGT GATCATCTTG GTCAACACGC CTACCCAGTA
CAGTATCATG 600

ATCCAGAATG TGGATGTTTA TGACGAAGGT CCGTACACCT GCTCTGTGCA
GACAGACAAT 660

CACCCCAAAA CCTCCCGGGT CCACCTCATA GTGCAAGTTC CTCCCCAGAT
AATGAACATC 720

TCTTCAGACA TTACTGTGAA TGAGATAAGC AGTGTGACCT TGTTATGTCT
CGCAATTGGC 780

AGACCAGAAC CAACAGTGAC ATGGCGACAC CTGTCAGTCA AGGAAGGCCA
GGGCTTTGTG 840

AGTGAAGATG AATACCTGGA AATCTCAGAC ATCAAACGCG ACCAATCTGG
AGAGTATGAG 900

TGCAGCGCCT TGAATGATGT CGCTGCACCT GATGTTCGGA AAGTAAAAAT
CACTGTAAAC 960

TATCCTCCCT ATATCTCAAA AGCGAAGAAC ACTGGCGTTT CAGTAGGCCA
GAAGGGCATC 1020

CTGAGCTGTG AAGCCTCTGC TGTCCCCATG GCTGAATTCC AGTGGTTCAA
GGAAGATACC 1080

AGGTTAGCCA CTGGCCTGGA TGGCGTGAGA ATTGAGAACA AAGGCCGCAT
ATCCACTTTG 1140

ACTTTCTTCA ATGTCTCAGA GAAGGATTAT GGGAACTATA CCTGTGTGGC
CACAAACAAG 1200

CTTGGGAACA CCAATGCCAG CATCACCCTG TATGGGCCTG GAGCAGTCAT
TGATGGTGTA 1260

AACTCGGCCT CTAGAGCACT GGCTTGTCTC TGGCTCTCAG GGACCTTCTT
TGCCCACTTC 1320

TTCATCAAGT TTTGATAAGA AACCTTAGGT CCTCTGAGCA TCGCCTGCTT
CTCCATATCA 1380

CAGACTTTAA TCTACACTGC GGAGGGGCAA ACCAGTTTGG GCTTTCTTTT
GGTTATTTTT 1440

TTGTTCTTCT TGACTGTTTA GTTTTTTGGT TTGATTTCTG GGATTTTCAA
TTTGATTTGT 1500

TTTTCTTTTT TCGTTTGAAT GGACCGGGGT TGGGGGTTGG GATGGGCAGG
GTTCTACCAC 1560

GAGTAGGATA ATCAGGTATT GGTGGGCCCC CAAATGGAAT ATATTCCTGC
TACCTTGGCC 1620

TTCCTTTTCT CTACTTCTCT TCCTACCACC ATTAACACAC AAACACACAA
GCACACGCCC 1680

TAAAGATGGC CTAAAAAATG TCCCATGACA CGCACCCTGA AGGTACAACT
TGGCCCACAG 1740

TGCAGTACAC AATAAGAGTT GCATCTACAT TTCCTGTTTT CTTTGTCCTT
TAAGTTTTCA 1800

ATAAGACAGT TTAAAAAGAG CACATCCTTA TCCCTATGTT TGTATCACCT
ATCCCATTAA 1860

GCTGCACACC TTTTCTAAGA AACTTTCTTA CTACATCCTC AATGTGCACA
CATGTACATT 1920

CTCATAAAAA TTTTACCATC TTCTCTGGCC ATTCTGCTTA ATCTGCATTT
TCCACTAGCA 1980

GTAAGACTTA CAGGCTTGAT GAAATATACA TGTATCCAAA GCTACAAATT
TAGAAGTCAC 2040

GAGGGAAGTC TATCTAGGGG TAGTACTTCC CTTAAAATGC TAATGCAACT
CATAAAAAAG 2100

TGATCAATAG CTAGCTAATT ATATCAAGCT ATCAAAGCAA TCATATTATT
ATCCAGTTAC 2160

TCAATTGATT TCTCTCTCC
2179

It is Claimed:

1. A transgenic, non-human animal all of whose germ cells and somatic cells contain an oligonucleotide sequence introduced into said animal, or an ancestor of said animal, at an embryonic stage, said oligonucleotide sequence expressible in the animals and effective to alter the animal's response to a narcotic analgesic.

2. The animal as in claim 1 wherein the altered response to a narcotic analgesic is a reduced sensitivity to the narcotic analgesic.

3. The animal as in claim 1 or 2 wherein the narcotic analgesic is morphine, a morphine analogue, an endogenous opioid, or a synthetic opioid peptide.

4. The animal as in claim 1 or 2 wherein the oligonucleotide sequence forms a transcriptional unit expressible in the animal as a messenger RNA compound capable of hybridizing to endogenous mRNA for an opioid binding protein.

5. The animal as in claim 4 wherein the oligonucleotide sequence is double or single stranded and is selected from all or a portion of the sequences of the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, but wherein the selected sequence, or sequence portion, is reversed with respect thereto.

6. The animal as in claim 4 wherein the oligonucleotide sequence selected has at least about 12 bases.

7. The animal as in claim 4 wherein th oligonucleotide sequence is selected from all or a portion of the first 500 bases of SEQ ID NO:1.

8. A method of providing a cell culture comprising:

providing a transgenic, non-human animal all of whose germ cells and somatic cells contain an oligonucleotide sequence introduced into said animal, or an ancestor of said animal, at an embryonic stage, said oligonucleotide sequence expressible in the animal and effective to alter the animal's response to a narcotic analgesic; and

culturing one or more somatic cells from said animal.

9. An oligonucleotide construct comprising a segment corresponding to all or part of the first about 500 bases of SEQ ID NO:1, but reversed with respect thereto, the segment being sufficient in size to be capable of hybridizing with endogenous mRNA for an opioid binding protein.

10. The construct as in claim 9 wherein the segment has at least about 12 bases.

11. The construct of claim 9 wherein said segment is operatively linked to an expression vector.

12. The construct as in claim 11 wherein the expression vector is a mammalian or retroviral expression vector.

13. The construct as in claim 9 wherein at least a portion of the normal phosphodiester backbone

has been modified with an analogue effective to limit degradation *in vivo*.

14. The construct as in claim 13 wherein the modification is by means of a methylphosphonate, a phosoramidate, a phosphorothioate, a 2'-o-alkyl, a polyamide (2-aminoethylgylcine) or a modification of ribose to a cycloalkane or partially unsaturated cycloalkane.

15. The construct as in claim 9 wherein the segment has a complementary strand associated therewith.

16. The construct as in claim 9 or 15 wherein the bases of the segment correspond to RNA.

17. A diagnostic or therapeutic agent comprising an oligonucleotide with at least about 12 bases when single stranded and about 12 base pairs when double stranded and having a backbone, the oligonucleotide defining a sequence of DNA bases or RNA bases analogous to all or a portion of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, but in the antisense direction thereof.

18. The agent as in claim 17 wherein at least a portion of the backbone has been modified effective to limit enzymatic degradation.

19. The agent as in claim 17 wherein the fragment is capable of hybridizing to mRNA for an opioid binding protein.

20. The agent as in claim 17 including a means for therapeutically administrating the oligonucleotide to a patient.

AMENDED CLAIMS

[received by the International Bureau on 30 August 1993 (30.08.93); original claims 1,4,9 and 10 amended; other claims unchanged (3 pages)]

1. A transgenic, non-human animal all of whose germ cells and somatic cells contain an oligonucleotide sequence introduced into said animal, or an ancestor of said animal, at an embryonic stage, wherein expression of the oligonucleotide sequence in the animals affects the animal's response to a narcotic analgesic.

2. The animal as in claim 1 wherein the altered response to a narcotic analgesic is a reduced sensitivity to the narcotic analgesic.

3. The animal as in claim 1 or 2 wherein the narcotic analgesic is morphine, a morphine analogue, an endogenous opioid, or a synthetic opioid peptide.

4. The animal as in claim 1 or 2 wherein the oligonucleotide sequence forms a transcriptional unit in the animal as a messenger RNA compound which hybridizes to endogenous mRNA for an opioid binding protein.

5. The animal as in claim 4 wherein the oligonucleotide sequence is double or single stranded and is selected from all or a portion of the sequences of the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, but wherein the selected sequence, or sequence portion, is reversed with respect thereto.

6. The animal as in claim 4 wherein the oligonucleotide sequence selected has at least about 12 bases.

7. The animal as in claim 4 wherein the oligonucleotide sequence is selected from all or a portion of the first 500 bases of SEQ ID NO:1.

8. A method of providing a cell culture comprising:

providing a transgenic, non-human animal all of whose germ cells and somatic cells contain an oligonucleotide sequence introduced into said animal, or an ancestor of said animal, at an embryonic stage, said oligonucleotide sequence expressible in the animal and effective to alter the animal's response to a narcotic analgesic; and

culturing one or more somatic cells from said animal.

9. An oligonucleotide construct comprising a segment corresponding from about 12 to about 500 bases of SEQ ID NO:1, but reversed with respect thereto, the segment being sufficient in size to hybridize with endogenous mRNA for an opioid binding protein.

10. The construct as in claim 9 wherein the segment is effective to suppress expression of an opioid receptor component gene.

11. The construct of claim 9 wherein said segment is operatively linked to an expression vector.

12. The construct as in claim 11 wherein the expression vector is a mammalian or retroviral expression vector.

13. The construct as in claim 9 wherein at least a portion of the normal phosphodiester backbone

has been modified with an analogue effective to limit degradation *in vivo*.

14. The construct as in claim 13 wherein the modification is by means of a methylphosphonate, a phosoramidate, a phosphorothioate, a 2'-o-alkyl, a polyamide (2-aminoethylgylcine) or a modification of ribose to a cycloalkane or partially unsaturated cycloalkane.

15. The construct as in claim 9 wherein the segment has a complementary strand associated therewith.

16. The construct as in claim 9 or 15 wherein the bases of the segment correspond to RNA.

17. A diagnostic or therapeutic agent comprising an oligonucleotide with at least about 12 bases when single stranded and about 12 base pairs when double stranded and having a backbone, the oligonucleotide defining a sequence of DNA bases or RNA bases analogous to all or a portion of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, but in the antisense direction thereof.

18. The agent as in claim 17 wherein at least a portion of the backbone has been modified effective to limit enzymatic degradation.

19. The agent as in claim 17 wherein the fragment is capable of hybridizing to mRNA for an opioid binding protein.

20. The agent as in claim 17 including a means for therapeutically administrating the oligonucleotide to a patient.

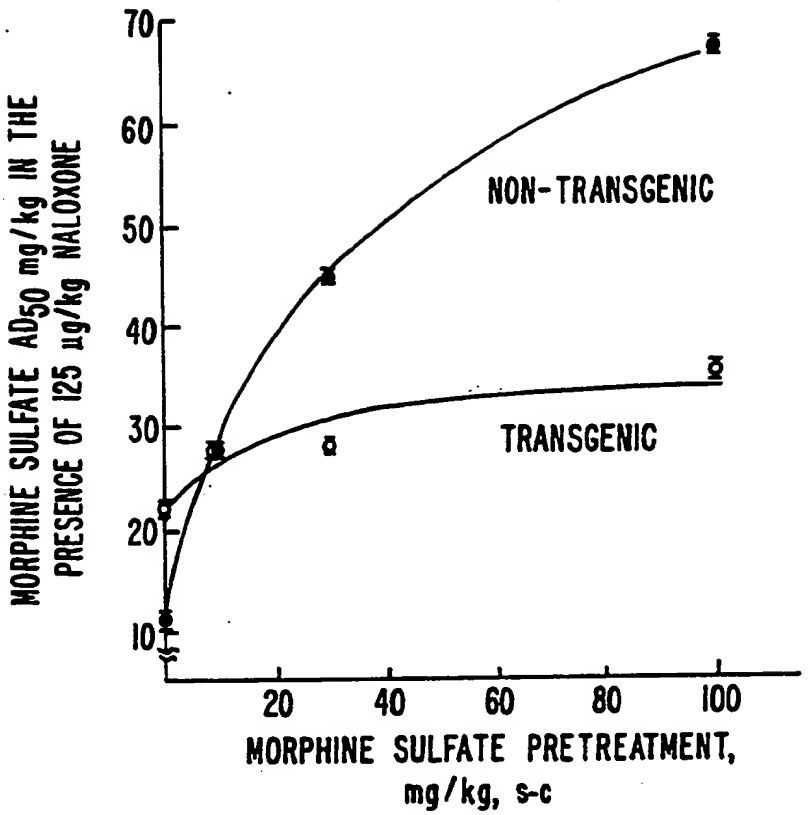
MORPHINE SULFATE AD50 mg/kg IN THE PRESENCE OF 125 µg/kg NALOXONE
70
60
50
40
30
20
10
NON-TRANSGENIC
TRANSGENIC
20
40
60
80
100
MORPHINE SULFATE PRETREATMENT, mg/kg, s-c

FIG. 1.

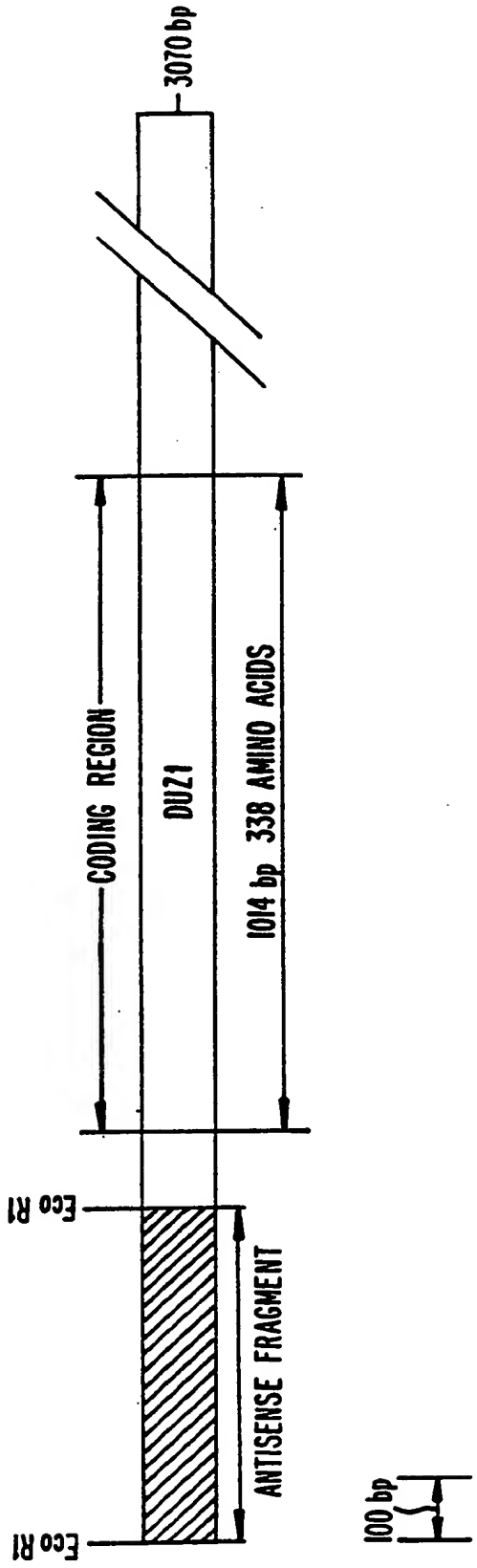
3070 bp
CODING REGION
DUZ1
1014 bp 338 AMINO ACIDS
Eco RI
ANTISENSE FRAGMENT
Eco RI
100 bp

FIG. 2.

THIS PAGE BLANK (USPTO)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02913

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00; C07H 15/12, 17/00; A61K 31/70
US CL :800/2; 536/24.5; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 536/24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,087,571 (Leder et al) 11 February 1992, col. 9, lines 11-21.	8
Y	Advances in Genetics, volume 24, issued 1987, G. Scangos et al, "Gene Transfer into Mice", pages 285-322, see entire document.	1-16
Y	Proceedings National Academy of Sciences, volume 78, no. 11, issued November 1981, S.V. Suggs et al, "Use of Synthetic Oligonucleotides as Hybridization Probes: Isolation of Cloned cDNA Sequences for Human ß2-Microglobulin", pages 6613-6617, especially page 6613, col. 1, lines 9-22.	1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 July 1993	Date of mailing of the international search report 13 JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer DEBORAH CROUCH, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02913

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 83, issued June 1986, T. M. Cho et al, "Purification to Apparent Homogeneity of a mu-Type Opiod Receptor from Rat Brain", pages 4138-4142, see entire document.	1-16
Y	Anti-Cancer Drug Design, volume 2, issued 1987, P.S. Miller et al, "A New Approach to Chemotherapy Based On Molecular Biology and Nucleic Acid Chemistry: Mutagen (Masking Tape for Gene Expression), pages 117-123, see entire document.	9-20

INTERNATIONAL SEARCH REPORT

Int[illegible]ati nal application No.
[illegible]T/US93/02913

Box I Observations where certain claims were f und unsearchable (Continuation f item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)

I. Claims 1-8, drawn to a first product and a first method, classified in Class 800/2.
II. Claims 9-20, drawn to a second product, classified in 536, subclass 24.5.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report cov rs only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ N protest accompanied the payment of additional search fees.